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Dermal fibroblasts from pseudoxanthoma elasticum patients have raised MMP-2 degradative potential

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Abstract

Cultured fibroblasts from the dermis of normal subjects and of Pseudoxanthoma elasticum (PXE) patients were analysed for enzyme activity, protein and mRNA expression of metalloproteases (MMP-2, MMP-3, MMP-9, MT1-MMP) and of their specific inhibitors (TIMP-1, TIMP-2 and TIMP-3). MMP-3, MMP-9 and TIMP-3 mRNAs and proteins failed to be detected in both the medium and the cell layer of both controls and PXE patients. MMP-2 mRNA was significantly more expressed in PXE than in control cell lines, whereas MT1-MMP, TIMP-1 and TIMP-2 mRNAs appeared unchanged. MMP-2 was significantly higher in the cell extracts from PXE fibroblasts than in control cells, whereas differences were negligible in the cell medium. Data suggest that PXE fibroblasts have an increased proteolytic potential, and that MMP-2 may actively contribute to connective tissue alterations in this genetic disorder.

Keywords: Matrix degradation; MMP-2; Pseudoxanthoma elasticum; Skin fibroblast; TIMP

1. Introduction

Pseudoxanthoma elasticum (PXE) is an inherited disorder mainly characterized by progressive disruption and mineralization of elastic fibres [1]. The PXE gene belongs to the ABC-binding cassette family (ABCC6) and encodes for the transmembrane transporter Multidrug Resistance Protein 6 (MRP6), whose biological function is still unknown [2–4]. Ultrastructural, cytochemical and immunocytochemical studies on PXE skin biopsies suggested that PXE lesions are also characterized by the deposition of a series of extracellular matrix (ECM) constituents and/or fragments [5,6] that were able to remain enclosed within elastic fibres during their formation [7]. In favour of this hypothesis, PXE skin fibroblasts in vitro have been shown to produce proteoglycans (PGs) with abnormal hydrophobic interaction properties and abnormal electrophoretic mobility [8,9]. Moreover, PXE fibroblasts in vitro have been shown to exhibit proteolytic activities [10,11] that might lead to ECM degradation products such as those described in the extracellular space of the PXE dermis in the form of huge aggregates of filaments positive for a series of matrix constituents [6]. Therefore, abnormal constituents and/or degradation products could favour connective tissue alterations typical of PXE, among which the formation of mineral precipitates, as also suggested by the uptake of calcium by PXE skin biopsies when incubated in the presence of high calcium concentration [12].

Proteases are known to play a major role in the homeostasis of connective tissues whose integrity depends on the balance between the synthesis and degradation of their various components [13]. Elastolytic activities in PXE have been described since the mid-sixties [14] and

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confirmed very recently by Annovazzi et al. [15], who determined the extent of degradation of elastin by measuring and comparing the amount of desmosines in plasma and urine of PXE patients, healthy carriers and normal subjects. The urinary excretion of desmosines was significantly higher in PXE patients than in controls, the values for healthy carrier being intermediate between those of PXE patients and controls. A very similar trend between patients and their relatives was observed also in plasma.

However, besides elastases [11,16], very few data are available on the characterization of other proteinases that may be responsible for the degradation of connective molecules registered in PXE. To highlight their potential contribution to the disease, both expression and activity of gelatinases MMP-2, MMP-3 and MMP-9, and of their activator MT1-MMP, together with the inhibitors TIMP-1, TIMP-2 and TIMP-3, were analysed in the medium and in the cell layer of PXE dermal fibroblasts, and compared to that of age- and sex-matched controls.

2. Experimental procedures

2.1. Patients and biopsies

Dermal biopsies from the neck or axilla were obtained after informed and signed consent from six controls (mean age 37±12 years) and from 10 subjects affected by PXE (mean age 36±13 years). From each biopsy sample, fibroblast cultures were established and cells grown as already described [17]. Fibroblasts up to the eighth passage were used. Data from three to five sets of experiments for each parameter are reported. In each experiment, measurements from each cell line were performed in duplicate. Student's *t*-test was used for comparison of data.

2.2. Cell culture

Unless otherwise specified, cells were seeded in 25-cm^2 flasks at a density of 3×10^5 cells with 5 ml of Dulbecco's modified Eagles medium (DMEM) containing 10% FCS. In a set of experiments, pretreated FCS was used, prepared as follows in order to remove gelatinolytic activities [18]: FCS was mixed with 20% v/v of Gelatin-Sepharose (Amersham Pharmacia, Biotech, UK), mixed gently at 4 °C for 2 h, centrifuged at 100×*g* for 5 min; the supernatant and pellet were analysed by gelatin zymography to verify removal of all gelatinases from the serum.

After 24 and 48 h, the medium was removed and replaced. After 3 days the cells were washed twice in PBS to remove residual FCS, and incubated for 24 h with 5 ml of serum-free DMEM without red phenol, or with DMEM supplemented with gelatinase-deprived FCS (for zymography). The culture supernatants were harvested and cellular debris removed by centrifugation at 100×*g* for 10 min. The medium was stored at −80 °C. Cells were removed from the substratum with 2 mM EDTA in PBS without calcium and magnesium for 10 min at 37 °C; EDTA was blocked by addition of the same amount of PBS with calcium and magnesium. After a rapid centrifugation, the pellets were lysed on ice with 0.5–1 ml of 1% Triton X-100, 1 mM PMSF, 0.5% NP-40, 1 mM EDTA, 150 mM NaCl, 10 mg/ml aprotinine in 50 mM Tris–HCl, pH 7.4. Cells lysates were centrifuged at 14,000×*g* for 15 min at 4 °C and the supernatants harvested and stored at −80 °C until used.

Cell number was evaluated on flasks grown in parallel by the Neubauer chamber. The protein content was measured by the Lowry method.

2.3. RNA extraction

Cells were plated at a density of 1.0×10^6 in 10-cm^2 Petri dishes with 10 ml of DMEM growth medium. After 24 h the medium was removed and replaced. After 5 days, the RNeasy Protocol^R (RNeasy Mini Kit, Quiagen, Bothell, WA, USA) was applied. The medium was removed and the cell layer washed with 10-ml PBS. Cells were lysated in 600 μl of Buffer RLT (RNeasy Protocol^R), scraped and passed at least three times through a 20-G needle syringe (0.9 mm diameter). Six-hundred microliters of 70% ethanol was added to the homogenized lysate and carefully mixed by pipetting. The sample was added to RNeasy mini spin column seated in a 2-ml tube (RNeasy Protocol^R) and centrifuged for 15 s at ≥8000×*g*. The pellet was washed with Buffer RW1 and Buffer RPE, and RNA was eluted with RNAse-free water. Total RNA was stored at −80 °C. RNA yield and purity were checked by spectrophotometric determinations at 260 and 280 nm.

2.4. Northern blot analysis

For Northern blot analysis, 10 μg of total RNA was resolved by electrophoresis through 1% agarose/2.2 M formaldehyde gel and capillary-transferred in 20× SSC (0.15 M NaCl, 15 mM Na citrate, pH 7) to nylon membranes (Hybond-N, Amersham-Pharmacia, Amersham, UK). Northern blots were hybridized for 3 h using 1×10^6 cpm/ml labelled probes in 50% formamide, 5× SSC, 50 mM Na_2PO_4, 50 μg/ml sonicated salmon sperm DNA, 1× Denhardt's solution at 68 °C.

Probes were synthesized by PCR, using the primers and conditions listed in Table 1, and radiolabelled with [α-^{32}P]dCTP using a random primer labelling kit (Amersham-Pharmacia). Blots, hybridized using human probes for MMP-2, MMP-3, MMP-9, MT1-MMP, TIMP-1, TIMP-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were washed at room temperature in 0.1× SSC, 0.1% SDS, and exposed at −70 °C to MP-hyperfilm (Amersham-Pharmacia) with intensifying screens. On the same film, relative levels of the transcripts were quantified by an image analyser system with GelDoc 2000 and Quantity One software (Bio-Rad, Hercules, CA, USA).

Table 1
Oligonucleotide sequences and conditions for synthesis of probes by PCR

For			From–To
MMP-2	sense	5′-ACCTGGATGCCGTCGTGGAC-3′	(1800–1819)
	antisense	5′-TGTGGCAGCACCAGGGCAGC-3′	(2228–2247)
	annealing/amplif.	62 °C for 30 s/28 cycles	
MMP-3	sense	5′-GAACAATGGACAAAGGATACAAC-3′	(664–686)
	antisense	5′-AAATGAAAACGAGGTCCTTGCTAG-3′	(1103–1126)
	annealing/amplif.	60 °C for 30 s/25 cycles	
MMP-9	sense	5′-GGTCCCCCCACTGCTGGCCCTTCTACGGCC-3′	(1501–1530)
	antisense	5′-CCTTTCCCTCCTCACCTCCAC-3′	(2243–2263)
	annealing/amplif.	62 °C for 30 s/30 cycles	
MT1-MMP	sense	5′-CCGTTTCAACGAAGAGC-3′	(1419–1435)
	antisense	5′-TCAGACCTTGTCCAGCAG-3′	(1852–1869)
	annealing/amplif.	55 °C for 30 s/30 cycles	
TIMP-1	sense	5′-CAGCCATATGTGCACCTGTGTCCCACC-3′	(126–152)
	antisense	5′-CGGGGGATCCTCAGGCTATCTGGGACC-3′	(674–700)
	annealing/amplif.	57 °C for 75 s/28 cycles	
TIMP-2	sense	5′-TGCAGCTGCTCCCCGGTGCAC-3′	(349–369)
	antisense	5′-TTATGGGTCCTCGATGTCGAG-3′	(913–933)
	annealing/amplif.	62 °C for 30 s/27 cycles	
TIMP-3	sense	5′-GGCTCGAGGGCGTGCACATGCTCGCCCAGCCAC-3′	(92–123)
	antisense	5′-CTGGAATTCAGGGGTCTGTGGCATTGATGAT-3′	(637–666)
	annealing/amplif.	60 °C for 30 s/25 cycles	
GAPDH	sense	5′-ACCACAGTCCATGCCATCAC-3′	(601–620)
	antisense	5′-TCCACCACCCTGTTGCTGTA-3′	(1031–1050)
	annealing/amplif.	60 °C for 30 s/25 cycles	

For each species of mRNA analysed, the densitometric units were normalized with reference to the GAPDH control [19].

2.5. Zymography

For zymography, media (DMEM with gelatinase-deprived FCS) conditioned 24 h by the cells and clarified were mixed with 4×SDS reducing sample buffer (1:1 ratio v/v). Cell layer extracts were enriched in MMP activities by affinity chromatography on excess of Gelatin-Sepharose before zymography [20].

Zymography was performed using 10% (w/v) acrylamide gel copolymerised with either gelatin (1 mg/ml) to identify proteins with gelatinolytic activities or casein (1 mg/ml) to identify stromelysin (MMP-3). After incubation of the gel in an activating buffer and staining with 0.5% (w/v) Coomassie brilliant blue R-250, proteolytic activities were detected as clear bands against the blue background, indicating areas where gelatin or casein were degraded by the enzymes. The molecular weights of gelatinolytic bands were estimated by comparing their electrophoretic migration to that of protein standards (Bio-Rad). The area of gelatinolytic bands was evaluated by a GS700 densitometer (Bio-Rad) and showed a linear increase with increasing amounts of standard MMPs (from 0.2 to 1 μg, regression coefficient (r^2)=0.90±0.04) [21]. As positive control of MMP inhibition, the gels were incubated in the presence of 20 mM Na_2-EDTA, which completely prevented gelatin digestion.

2.6. Immunoblotting

Immunoblot analysis of MMP-2, MT1-MMP, TIMP-1, TIMP-2 was performed after SDS-PAGE (10% and 12% polyacrylamide, respectively), and Western transfer to PVDF membranes using a semi-dry transfer cell (Bio-Rad) at 15 V/cm^2 for 20 min. The identification was then performed with specific anti-human anti-sera (sheep host) (1:25,000) (The Binding Site, Birmingham, UK) in the case of MMPs and with specific anti-human anti-sera (rabbit host; dilution 1:25,000) (Chemicon, Temecula, CA, USA) in the case of TIMPs, using chemiluminescence (Super Signal, Pierce, Rockford, IL, USA). The antibodies against MMP-2 react with both activated and latent forms of these enzymes [22,23].

3. Results

3.1. RNA expression

Fig. 1A shows mRNA expression for MMP-2, MT1-MMP, TIMP-1, TIMP-2 and GAPDH. MMP-2 mRNA was significantly higher in the PXE than in the control group, whereas MT1-MMP, TIMP-1 and TIMP-2 mRNA expression exhibited nonsignificant differences between normal and pathological fibroblasts (Fig. 1B). It should be mentioned that, under our experimental conditions, MMP-9 and TIMP-3 mRNAs could not be detected.

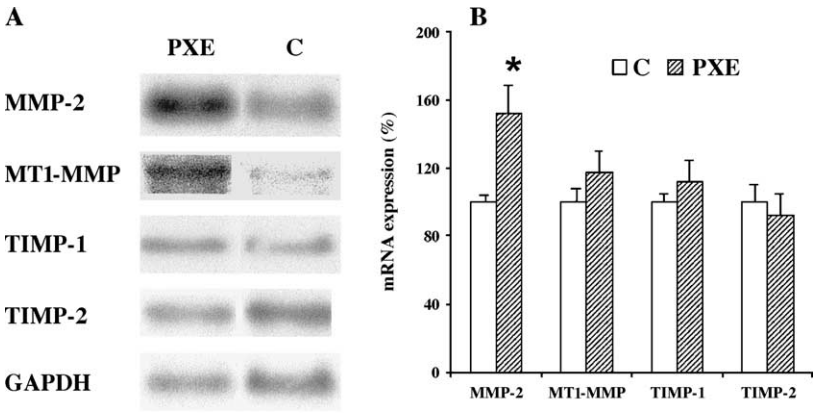

Fig. 1. (A) A representative Northern blot of MMP-2, MT1-MMP, TIMP-1, TIMP-2 and GAPDH as internal standard from control (C) and Pseudoxanthoma elasticum (PXE) fibroblasts. (B) Data from three experiments, performed each time with three and four different control and PXE fibroblast cultures, respectively, are reported as percentage variations of mean values±S.D. of densitometric analyses normalized against GAPDH. $^{**}P \leq 0.006$ (PXE versus control).

3.2. Zymography and immunoblotting

On the basis of the data obtained by zymography, neither stromelysin (MMP-3) (data not shown) nor MMP-9 (Fig. 2D) was detectable in the medium or in the cell layer of either control or PXE fibroblasts. By contrast, a gelatinolytic band at about 72 kDa, corresponding to pro-MMP-2 control, was present in the cell layer from normal fibroblasts and was

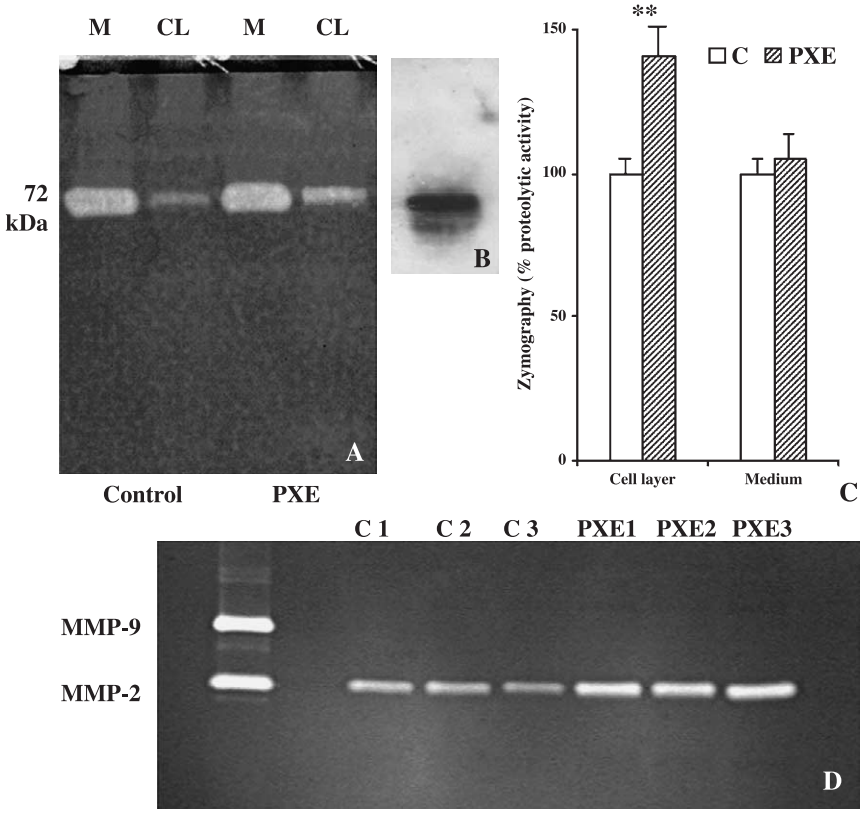

Fig. 2. Gelatinolytic areas formed by cell extracts (CL) and by culture media (M) of control and PXE fibroblasts in one representative experiment are shown in panel A. Immunoblotting of culture medium from PXE fibroblasts with anti-MMP2 antibodies (panel B) confirms that the identity of the proteolytic activity in panel A is pro-MMP-2, and also shows its activated form (lower band). Densitometric evaluations of five experiments, performed each time with three different control and PXE cell cultures, are reported in panel C (mean values±S.D.). A total number of six different control and 10 PXE fibroblast cultures have been analysed. The gelatinolytic activity, expressed as percentage of control activity, is significantly higher in the cell layer of PXE fibroblasts compared to controls, whereas differences are negligible in the culture media. $^{**}P \leq 0.002$ (PXE vs. control). Panel D shows the zymography of a representative experiment demonstrating the increased activity of the cell layer from three different in vitro cultured PXE fibroblast cell lines compared to control cells. Zymography loads were normalized for number of cells.

even more evident in the cell layer from PXE fibroblasts (Fig. 2A). The identification was confirmed by specific antiserum against MMP-2, which recognises both the zymogen and activated forms of the enzyme (Fig. 2B).

Densitometric analyses of six control and 10 PXE cell cultures, analysed in five different experiments, showed that the gelatinolytic band corresponding to MMP-2 was significantly more pronounced in the cell layer of PXE samples with respect to controls, whereas differences were negligible in the culture medium (Fig. 2C).

4. Discussion

The present investigation has been undertaken with the aim of exploring the role of metalloproteases and of their inhibitors in PXE extracellular matrix degradation. Data demonstrate that cell extracts from PXE fibroblasts express significantly higher MMP-2 mRNA as well as MMP-2 enzymatic activity compared to fibroblasts from normal subjects.

MMP activity is known to be regulated at three different levels: gene transcription, posttranslation activation of zymogens and interactions of secreted MMPs with inhibitors [24]. The present data indicate that PXE fibroblasts possess a latent higher proteolytic potential that can be activated by local factors and/or changes in the cellular microenvironment, consistent with the occurrence of heterogeneous ECM alterations in PXE patients.

Therefore, the present findings further expand the panel of proteolytic activities reported in the literature as instrumental in PXE connective tissue alterations [10,11]. MMP-2 has been shown to cleave chondroitin sulfate proteoglycans, decorin, fibronectin, osteonectin, laminin and IL1β, to degrade dermal collagen and elastin fibres during skin aging [25] and to favour the development of calcific aortic stenosis [26].

Besides elastic fibre mineralization, abnormalities in several other matrix molecules, such as collagens and glycosaminoglycans, have been described in PXE lesions, therefore the higher than normal MMP-2 activity would be in agreement with the complex disarrangement of the whole ECM in PXE [27]. It could be hypothesised that the protease/gelatinase-inhibitor imbalance in PXE may lead to progressive degradation of ECM constituents that would accumulate in the extracellular space in the form of filamentous aggregates [5,6]. Moreover, degradation products of either normal matrix molecules [28] or proteins and proteoglycans abnormally produced by PXE cells [7–9] may remain trapped within the elastic fibres, thus contributing to the ion precipitation typical of PXE [1,29].

It should be mentioned that calcium-containing deposits that are present in PXE mineralizations could exert different biological roles such as activation of signal transduction pathways, nuclear transcription factors, cell duplication, and metalloprotease synthesis [30,31]. These effects are consistent with the high proliferation index and the altered cell–matrix interactions observed in PXE cultured fibroblasts [17]. In addition, changes in cell–matrix interactions and cell spreading have been reported to alter MMP secretion and activation [32]. Since it has been shown that one of the mechanisms of pro-MMP-2 activation involves the zymogen forming a complex at the cell surface with MT1-MMP and TIMP-2, clustering of α2β1 and αvβ3 integrins, for instance, may sequester pro-MMP-2 within the focal adhesion and prevent it from interacting with its cell-surface-associated converting enzyme MT1-MMP [33]. Therefore, the local disconnection between cells and ECM, observed in cultured PXE fibroblasts [17], could favour the production of active MMP-2. In addition, MMP-2 activity may be modulated also by local factors such as fluctuations in intracellular pH, by the presence of metabolites such as oxidized glutathione and estradiol [34] or by the formation of reactive oxygen species (ROS) as observed in coronary artery disease [35] and in cardiac fibroblasts [36]. Therefore, changes in the cellular microenvironment, due to the absence and/or misfunctioning of the transporter MRP6, may trigger the activation of the degradative potential expressed by PXE fibroblasts. Moreover, the possible involvement of estradiol in MMP-2 activation may further support the observation that females appear to be more affected than man by PXE [1].

In conclusion, the present study shows that MMP-2 gelatinase is augmented in the cell layer but not in the culture medium of fibroblasts from PXE patients, suggesting that, at least in vitro, the degradative tool is more concentrated at the membrane level. Data indicate also that local factors may be responsible for the complete activation of MMP-2 in the extracellular space. Furthermore, the significant increase of mRNA for MMP-2 in PXE fibroblasts suggests the occurrence of altered pre-translational regulatory mechanisms.

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